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High Invasive Melanoma Cells Induce Matrix Metalloproteinase-1 Synthesis in Fibroblasts by Interleukin-1α and Basic Fibroblast Growth Factor-Mediated Mechanisms

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Tumor invasion and metastasis of melanoma have been shown to require proteolytic degradation of the extracellular environment, achieved primarily by enzymes of the matrix metalloproteinases (MMP) family. Increased enzyme activity is localized at the border of tumor cells and the adjacent peritumoral connective tissue, emphasizing the crucial role of tumor–stroma interactions in the regulation of MMP activity. To analyze whether direct cell–cell contacts of melanoma cells and stromal fibroblasts or whether soluble factors, secreted by melanoma cells are involved in the regulation of MMP, we used different *in vitro* co-culture systems. Both direct and indirect co-cultures of high invasive BLM melanoma cells and human dermal fibroblasts resulted in an induction of pro-MMP-1 synthesis. Medium conditioned by BLM cells strongly induced pro-MMP-1 synthesis in fibroblasts, indicating the importance of diffusible factors for this induction. Competition by recombinant human interleukin (IL)-1 receptor antagonist, neutralizing IL-1α and basic fibroblast growth factor (bFGF) antibodies, resulted in a concentration-dependent reduction of pro-MMP-1 synthesis. Taken together, our results indicate an essential role for soluble factors, mainly IL-1α and bFGF, in the stimulation of dermal fibroblasts by human melanoma cells to secrete MMP-1.

Key words: bFGF/dermal fibroblasts/IL-1α/matrix metalloproteinases/melanoma
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During invasion and metastasis of melanoma, tumor cells detach from the primary tumor, migrate, and penetrate several structural barriers, e.g. basement membranes and the interstitial connective tissue, and invade the surrounding connective tissue of the dermis. A series of studies revealed that tumor cells are not individual units within a tumor, but rather depend on the cross-talk with neighboring stromal cells and matrix components (Hart, 1982; Fidler, 1983; Liotta *et al*, 1983; Nicolson, 1987). Of particular importance are interactions between melanoma cells, the extracellular environment that includes different matrix constituents, neighboring fibroblasts, and microvascular endothelial cells. Although it is well documented that in embryonic development various growth factors, cytokines, matrix proteins, and proteinases act as crucial regulators of mesenchymal–epithelial cross-talk, increasing evidence suggests that these regulators are also important in the regulation of tumor growth. Recent studies with stroma-oriented cancer therapy were remarkably successful and are considered as a particularly promising novel approach for the treatment of malignancy (Ferrara *et al*, 2004). Nevertheless, the knowledge in this area is still rudimentary.

Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IL, interleukin; IL-1RA, IL-1 receptor antagonist; MMP, matrix metalloproteinase; TGF-β1, transforming growth factor β1

The best-characterized proteinases in the degradation of matrix components are matrix metalloproteinases (MMP) (Sternlicht and Werb, 2001; Woessner, 2002; Somerville *et al*, 2003). Besides regulation of pro-MMP/MMP conversion, the enzyme activity is controlled by the endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMP). We have recently shown that the activation of pro-MMP-2 by MT1-MMP induced by the contact of melanoma cells with fibrillar type I collagen is differentially regulated, in high and low invasive cells, by the levels of TIMP-2 (Kurschat *et al*, 1999). Others have shown that upregulation of TIMP-1 resulted in suppression of *in vitro* invasive capacity (Khokha *et al*, 1992), underlining the importance of MMP in tumor cell invasion.

In addition, cytokines and growth factors have been shown to affect cell adhesion and motility, proliferation, and matrix degradation (Dekker *et al*, 1997). Among them are interleukin (IL)-1, IL-8, transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (bFGF), which may stimulate, in a paracrine fashion, resident cells of the surrounding stroma, e.g. fibroblasts, endothelial cells, keratinocytes, and inflammatory cells leading to enhanced tumor invasion and angiogenesis (Coussens and Werb, 2002). Recent experiments demonstrated that transduction of the metastatic melanoma cell line SMEL (constitutively producing IL-1 at high levels) with IL-1 receptor antagonist (IL-1RA) results in significant reduction of tumor growth and metastasis to the lungs in nude mice, clearly indicating a role for

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IL-1RA in modulating IL-1 activities (Weinreich *et al*, 2003). Halaban *et al* (1988) have shown that the growth of melanoma cells is highly dependent on the production of bFGF and that the growth was significantly inhibited by injection of neutralizing anti-bFGF antibodies. Similarly, reduction of bFGF mRNA expression using an antisense approach could inhibit melanoma proliferation (Becker *et al*, 1989).

Several recent investigations have shown that in various human carcinomas, expression of MMP is localized to both tumor and stromal cells at the invading margin of the tumor, providing a mechanism for highly concerted degradation of the extracellular matrix. Increased expression of MMP-1 has been observed in several carcinomas with the most abundant expression of MMP-1 observed by the peritumoral stromal cells, but significant expression of MMP-1 has also been detected within the tumor by intratumoral endothelial cells (Johansson *et al*, 1997). Also in melanoma, MMP-1 mRNA and protein have been localized to stromal fibroblasts adjacent to the tumor cells, whereas tumor cells and fibroblasts distant from the tumor site stained negative for MMP-1 (Woolley and Grafton, 1980; Airola *et al*, 1999). In addition, it has been shown that melanoma patients with MMP-1- or MMP-3-positive metastases had a significantly shorter disease-free survival compared with patients with MMP-1 negative metastases, suggesting that MMP-1 and MMP-3 may have an important role in the formation of melanoma metastases (Nikkola *et al*, 2002). *In vitro*, A2058 melanoma cells were shown not to invade fibrillar type I collagen unless they were stimulated by fibroblast-conditioned medium (Benbow *et al*, 1999), suggesting that the stroma itself has a major impact on the invasive behavior of tumor cells.

In this work, we have addressed the importance of melanoma–stroma interactions for the induction of proteolytic enzymes, in particular of MMP-1, and the mediators involved in this cross-talk.

Results

Analysis of MMP-1 synthesis upon co-culture of human fibroblasts with melanoma cells In order to elucidate the role of direct or indirect interactions between human dermal fibroblasts and human melanoma cells in the regulation of pro-MMP-1 synthesis and activation, we cultured cells either in direct or indirect contact using transwell chambers. We examined two different melanoma cell lines, BLM and WM164, which we had characterized before in a skin equivalent culture model (Dennhofer *et al*, 2003). Although BLM cells were invading the de-epidermized skin, WM164 cells failed to do so, indicating differences in the ability of tumor cell invasion. MMP-1 protein levels were completely absent in BLM and WM164 melanoma cells, and low in monocultures of human dermal fibroblasts (Fig 1). A strong induction in pro-MMP-1 synthesis, as compared with fibroblast monoculture, was observed in both direct or indirect co-cultures with high invasive BLM melanoma cells (Fig 1*A*). In contrast, the co-culture of fibroblasts with low invasive WM164, either in direct or indirect contact, did not alter pro-MMP-1 synthesis (Fig 1*B*). To analyze whether the induction of pro-MMP-1 synthesis observed in the indirect co-culture

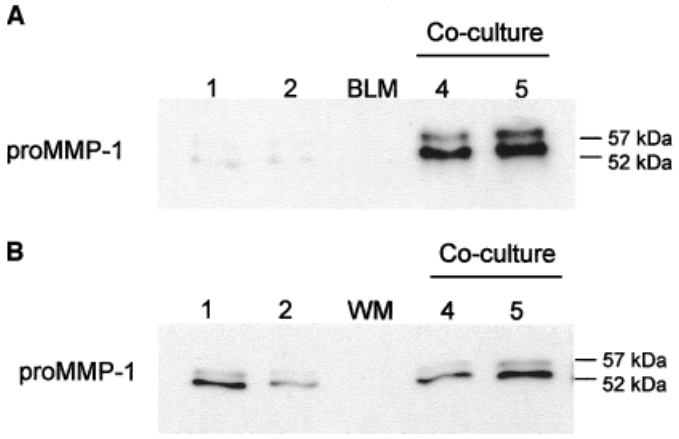

Figure 1
Pro-matrix metalloproteinase (MMP)-1 synthesis by fibroblasts is induced by direct and indirect co-culture of fibroblasts with high and low invasive melanoma cells. Human dermal fibroblasts (final cell number of 5×10^4 cells per cm^2, *lane 1*, and 2.5×10^4 cells per cm^2, *lane 2*) and melanoma cells (BLM and WM164; final cell number of 5×10^4 cells per cm^2 (*lane 3*) were cultured as monolayer. In addition, fibroblasts and melanoma cells were cultured in direct cell–cell contact in a 1:1 ratio (final cell number of 5×10^4 cells per cm^2, *lane 4*) and in a transwell system (5×10^4 cells per cm^2, *lane 5*). Co-cultures of BLM and fibroblasts are shown in (*A*), whereas co-cultures of WM164 and fibroblasts are shown in (*B*).

requires a cellular cross-talk or whether factors secreted by melanoma cells are, *per se*, sufficient to induce protease expression, we also cultured human fibroblasts in the presence of melanoma cell-conditioned medium. Treatment of fibroblasts with increasing concentrations of conditioned medium was associated with a 4-fold (25% BLM c.m.) and a 20-fold increase of MMP-1 protein (100% BLM c.m.), calculated after densitometric analysis as ratio *versus* untreated cells as control (Fig 2*A*). In contrast, conditioned media derived from the low invasive WM164 melanoma cells had no effect on pro-MMP-1 synthesis by fibroblasts (Fig 2*B*). These results indicate that soluble factors secreted by the high invasive melanoma cell line activate stromal fibroblasts and induce pro-MMP-1 synthesis.

Characterization of soluble factors involved in the induction of pro-MMP-1 synthesis In order to address which soluble mediators induce pro-MMP-1 synthesis, we firstly analyzed various cytokines and growth factors for their capability to induce MMP-1 expression in fibroblasts grown as monolayer culture for 72 h.

As positive control for the stimulation of the fibroblasts, we used phorbol 12-myristate 13-acetate (PMA) (0.4 nM), known to induce pro-MMP-1 synthesis in dermal fibroblasts (Xu and Clark, 1997). Whereas stimulation with recombinant IL-6 and epidermal growth factor (EGF) did not alter pro-MMP-1 synthesis, treatment with recombinant IL-1α, tumor necrosis factor (TNF)-α, and bFGF strongly induced pro-MMP-1 synthesis by 2-fold (Fig 3), indicating a potential role of these soluble factors in the interaction of melanoma cells and stromal fibroblasts. In contrast, treatment with recombinant TGF-β1 and activin reduced pro-MMP-1 synthesis to levels below untreated control.

Quantification of IL-1α in media conditioned by high or low invasive melanoma cells and by fibroblasts, using en-

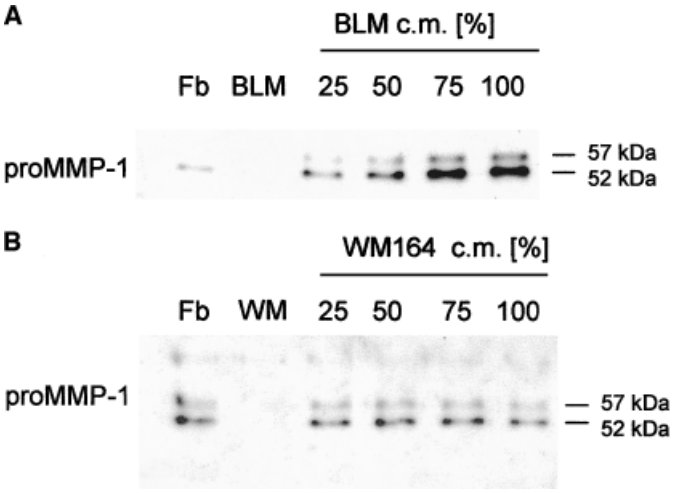

Figure 2
Western blot analysis of pro-matrix metalloproteinase (MMP)-1 expression. Human dermal fibroblasts (Fb; 5×10^4 cells per cm^2) were cultured in complete RPMI or in the presence of increasing amounts (% of the total volume of culture media) of medium conditioned by the melanoma cells (c.m.). (*A*) Treatment of Fb with conditioned medium from high invasive melanoma cells, BLM; (*B*) treatment of Fb with medium conditioned by low invasive WM164 melanoma cells.

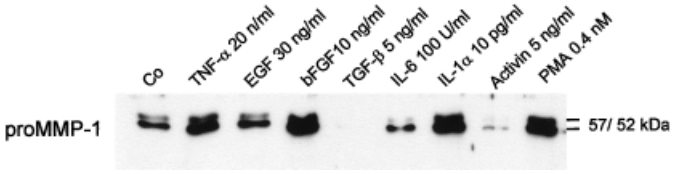

Figure 3
Effect of cytokines and growth factors on pro-matrix metalloproteinase (MMP)-1 synthesis by human dermal fibroblasts. Human dermal fibroblasts were cultured as monolayer in the absence (Co) or in the presence of recombinant tumor necrosis factor (TNF)-α (20 ng per mL), epidermal growth factor (EGF) (30 ng per mL), basic fibroblast growth factor (bFGF) (10 ng per mL), transforming growth factor-β1 (TGF-β1) (5 ng per mL), interleukin (IL)-6 (100 U per mL), IL-1α (10 pg per mL), Activin (5 ng per mL), and phorbol 12-myristate 13-acetate (PMA) (0.4 nM) as described in "Materials and Methods".

zyme-linked immunoassays, detected production of elevated concentrations of IL-1α (7.8 pg per mL) by the high invasive melanoma cell line BLM and very low production by the low invasive melanoma cells WM164 and by fibroblasts (Table I). Also, high amounts of bFGF were secreted by BLM melanoma cells (392.6 pg per mL), whereas low levels were detected in the medium conditioned by fibroblasts and by WM164 cells (Table I).

Inhibition of pro-MMP-1 synthesis by neutralizing IL-1α and bFGF signalling To analyze the direct involvement of these factors in the induction of pro-MMP-1 synthesis observed upon treatment of fibroblasts by melanoma cell-conditioned media, we performed competition assays in the presence of recombinant IL-1RA (2, 10, and 100 ng per mL), of neutralizing antibodies against IL-1α (5, 30, and 50 ng per mL) and against bFGF (10, 50, and 100 μg per mL). Treatment by either recombinant IL-1RA, or by neutralizing anti-IL-1α and by anti-bFGF antibodies resulted in a concentration dependent and nearly complete reduction of the 57/52 kDa pro-MMP-1 protein forms, corresponding to 70% inhibition as quantitated by densitometric analysis (Fig 4*A* and *B*). Treatment by a combination of both antibodies and IL-1RA, however, did not further decrease pro-MMP-1 expression, indicating that in addition to IL-1α and bFGF, other factors are involved in the induction of pro-MMP-1 synthesis (Fig 4*C*). In contrast, incubation of fibroblasts by neutralizing anti-IL-6 or anti-EGF antibodies failed to inhibit pro-MMP-1 synthesis (data not shown).

Table I. Quantification of IL-1α and bFGF in conditioned medium

	bFGF (pg per mL)	IL-1α (pg per mL)
Fb	77.7 ± 8.2	0
BLM	392.6 ± 55.1	7.8 ± 0.3
WM164	5.9 ± 8.3	0

Levels of bFGF and IL-1α in media, conditioned by Fb and melanoma cells, BLM, and WM164, were determined using a ELISA, as described under "Materials and Methods". The data represent the mean ± SD of triplicate experiments. Elevated levels of secreted bFGF and IL-1α were found in high invasive BLM melanoma cells, as compared with low invasive WM164 cells.
IL, interleukin; bFGF, basic fibroblast growth factor; Fb, fibroblasts.

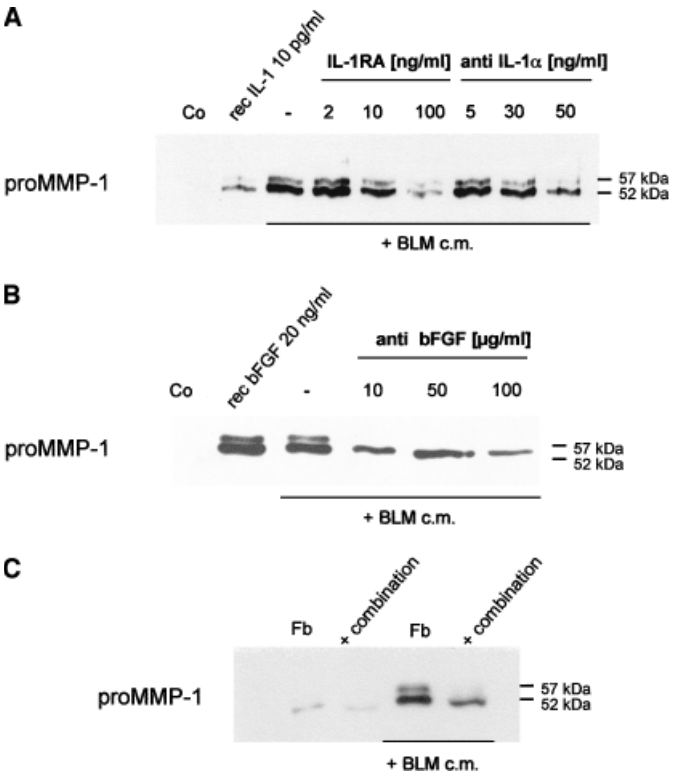

Figure 4
Role of interleukin (IL)-1α and basic fibroblast growth factor (bFGF) in the induction of pro-matrix metalloproteinase (MMP)-1 synthesis. Human dermal fibroblasts (Fb) were grown in complete RPMI (Co) or in the presence of 25% complete BLM cells conditioned medium (−). Co-cultures were incubated with increasing concentrations of: (*A*) recombinant IL-1 receptor antagonist (IL-1RA) (2, 10, and 100 ng per mL) or neutralizing IL-1α monoclonal antibodies (5, 30, and 50 ng per mL); (*B*) neutralizing bFGF monoclonal antibodies (10, 50, and 100 μg per mL); or (*C*) a combination of IL-1RA, anti-IL-1α, and anti-bFGF (100, 50, and 100 μg per mL, respectively). Additionally, Fb were stimulated with recombinant IL-1α (10 pg per mL) and recombinant bFGF (20 ng per mL).

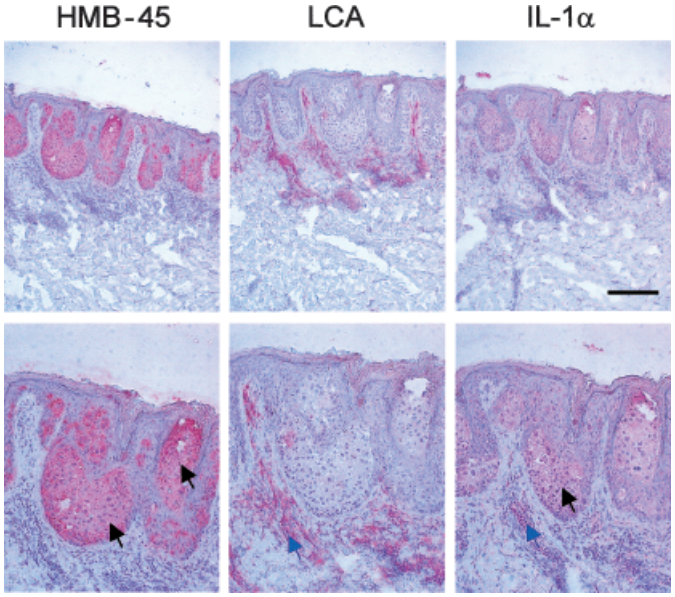

Figure 5
Distribution of interleukin (IL)-1α in human melanoma tissue. Cryosections (8 μm) of primary melanoma were stained with the HMB-45 antibody identifying melanoma cells (*black arrows*; as provided) and with anti-leukocyte common antigen (LCA) localizing the inflammatory infiltrate (*white arrows*; 1:100) and anti-IL-1α (10 μg per mL). Magnification, × 100 (*upper panel*) and × 200 (*lower panel*). *Scale bar* = 50 μm.

Distribution of IL-1α in human melanoma tissue Although bFGF expression by melanoma cells *in vivo* has already been studied (Reed *et al*, 1994; Kubo *et al*, 1998), we wanted to prove the relevance of IL-1α-mediated activation of peritumoral fibroblasts by melanoma cells *in vivo*. Therefore, we have analyzed cryosections of human melanoma specimen for the expression of IL-1α (Fig 5). Melanoma and inflammatory cells were identified by the immunohistochemical staining of the melanoma-specific antigen HMB-45 and the leukocyte common antigen (LCA), respectively. IL-1α was strongly expressed in the inflammatory cells (*blue arrows*) surrounding the tumor, but also by tumor cells (*black arrows*), indicating that both inflammatory and tumor cells are a source of IL-1α *in vivo*.

Discussion

A distinguishing feature of malignant cells is their capacity to invade the surrounding connective tissue, which is a prerequisite to metastasize through the vascular and lymphatic systems to distant organs. It is well established that tumor cell adhesion, mobility, proteolytic activities, cytokine secretion, and cell receptor expression play a crucial role in cancer invasion.

There is increasing evidence that protease expression is not restricted to neoplastic cells but may also originate from tumor-associated stromal cells. Using *in situ* zymography we have previously shown that the expression of the gelatinase MMP-2 is strongly increased in invasive melanomas when compared with benign melanocytic nevi and that this activity is localized mainly at the tumor–stroma border and in the peritumoral stroma (Kurschat *et al*, 2002).

MMP-1 transcripts were detected at the tumor periphery of advanced melanomas (Airola *et al*, 1999), indicating that MMP-1 may either be derived from the melanoma cells or the surrounding stromal cells. Wandel *et al* (2002) have shown that fibroblasts isolated from the peritumoral stroma of melanoma display increased synthesis of MMP-1, when compared with skin fibroblasts distant of the tumor site.

In our studies, we used two different melanoma cells, which are characterized by different invasive capacity in nude mice. Both melanoma cell lines did not express MMP-1 at either transcript or protein levels. In addition, we also observed no MMP-1 expression by freshly isolated melanoma cells either from primary tumors or from organ metastases (our unpublished observations). Using different co-culture systems, we detected increased pro-MMP-1 synthesis in fibroblasts by direct as well as indirect cell–cell contact with high invasive melanoma cells, whereas unstimulated control fibroblasts produced low amounts of this protease (Fig 1). Interestingly, melanoma cells isolated from a primary nodular melanoma and from an organ metastasis showed a similar strong induction of pro-MMP-1 synthesis in fibroblasts (data not shown). These findings clearly indicate that activation of stromal fibroblasts by high invasive melanoma cells is caused by soluble factors rather than by direct cell–cell contacts. Synthesis of pro-MMP-3, which was previously shown to activate pro-MMP-1 *in vitro* (Nagase *et al*, 1992), was also induced (data not shown). Under the conditions we used, however, we failed to detect increased levels of active MMP-1. Relatively high levels of the endogenous inhibitor, TIMP-1, in both fibroblasts and BLM cells (Kurschat *et al*, 1999; Zigrino *et al*, 2001), might inhibit the activity of MMP-3 thereby explaining the lack of activation of MMP-1 we observed. Alternatively, a three-dimensional environment, which resembles more closely the *in vivo* situation, might be needed for proper localization and activation of the precursor form.

Soluble factors were often suggested to mediate cellular communication between cancer and stromal cells (Ito *et al*, 1995; Wandel *et al*, 2002). Of several soluble factors shown to induce MMP-1 synthesis in dermal fibroblasts (Yamamoto *et al*, 2000; Dasu *et al*, 2003), here we identified IL-1α and bFGF as the main melanoma-derived factors inducing MMP-1 synthesis in fibroblasts. bFGF has recently been identified as a soluble factor responsible for the activation of fibroblasts (Wandel *et al*, 2002). In addition, Graeven *et al* (2001) showed that WM164 cells injected in nude mice failed to form metastases unless overexpressing bFGF or VEGF. We have measured very low levels of bFGF in culture media from these low invasive cells, compared with the high invasive melanoma cells (Table I), correlating with the lack of pro-MMP-1 induction by low invasive cells. The levels of different soluble factors and the expression of their receptors have been analyzed in human melanomas *in vivo* and *in vitro* (Herlyn, 1990). Analysis of specific transcripts in 21 melanoma cell and five melanocyte cultures showed that IL-1α and IL-1-β is expressed by melanoma cells but not by melanocytes (Mattei *et al*, 1994).

Besides the role of bFGF, the participation of IL-1α and IL-1-β in the invasiveness of malignant cells in experimental tumor models, as well as in cancer patients, has been extensively studied (Apte and Voronov, 2002). Fibrosarcoma

cells overexpressing IL-1α or IL-1β by transfection were found to become more aggressive when compared with mock-transfected cells (Song *et al*, 2003). Interestingly, using IL-1α and IL-1β knockout mice, these authors were able to show that local tumor or lung metastases of B16 melanoma cells were not observed compared with wild-type animals and that angiogenesis was impaired in these animals. The authors, however, primarily focused on the role of IL-1 released from tumor cells on the immune response and did not address changes within the peritumoral stroma (Voronov *et al*, 2003).

In our studies, stimulation of fibroblast monocultures with IL-1α and bFGF induced synthesis of pro-MMP-1, whereas treatment with TGF-β1 completely abolished the expression of pro-MMP-1. Other cytokines and growth factors, such as IL-6 and EGF, failed to alter the expression of this protease (Fig 3). Competition assays using either recombinant IL-1RA or neutralizing IL-1α and bFGF antibodies showed that these factors act in a direct paracrine fashion. Treatment with a combination of IL-1α and bFGF competitors (Fig 4*C*), however, failed to completely inhibit the expression of pro-MMP-1, thus indicating that other factors might be required to induce upregulation of pro-MMP-1 in fibroblasts by high invasive melanoma cells. Future work using array and proteomic technologies will facilitate the identification of additional factors involved in melanoma-fibroblast cross-talk.

In conclusion, our data support the idea that high invasive melanoma cells can activate stromal fibroblasts to produce MMP, which in turn facilitate degradation of the connective tissue and migration of tumor cells into the surrounding connective tissue.

Materials and Methods

Cell culture and preparation of conditioned media Primary human dermal fibroblasts were obtained by outgrowth from skin explants as previously described (Zigrino *et al*, 2001). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 U per mL each of penicillin and streptomycin in 5% CO_2, 37°C in a humidified atmosphere. Fibroblasts were passaged by trypsinization at a ratio of 1:2 every 5 d and used at passages 1–10.

The human high invasive and metastatic, BLM (Van Muijen *et al*, 1991), and the low invasive melanoma cells, WM164 (Herlyn, 1990), were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, non-essential amino acids, and 100 U per mL each penicillin and streptomycin (complete medium).

5×10^4 cells per cm^2 were seeded as monolayer for 48 h in complete RPMI 1640 medium. Cells were washed twice with phosphate-buffered saline (PBS) and medium replaced by serum-free RPMI 1640. After 24 h, the medium was collected, centrifuged at 2000 × *g* for 5 min, to remove cellular debris, and supernatants were transferred to new tubes. The total protein concentration in the conditioned medium was determined using the BCA Protein assay according to the manufacturer's instructions (Perbio Science, Bonn, Germany).

The study was approved by the local ethics committee and was in agreement with the Helsinki Declaration Principles.

Co-culture of melanoma cells and fibroblasts Fibroblasts and melanoma cells (5×10^4 cells per cm^2) were seeded either as monocultures or together in a 1:1 ratio as direct co-cultures. Alternatively, both cell types were cultured in indirect co-culture using transwell cell culture chambers (0.4 μm pore size; Becton Dickinson, Heidelberg, Germany). To analyze the effect of the melanoma-conditioned medium, fibroblasts were also cultured in the presence of 25%–100% melanoma-conditioned medium. All cell culture systems were incubated for 72 h in the above-described conditions.

Competition assays Fibroblasts were grown in complete RPMI medium in the presence of recombinant IL-1α (10 pg per mL, Tebu, Offenbach, Germany) or recombinant bFGF (20 ng per mL, Tebu) or containing 25% of BLM cells conditioned medium containing serum. Cells were treated with several concentrations of the recombinant IL-1RA (2, 10, and 100 ng per mL, R&D Systems, Wiesbaden, Germany), neutralizing IL-1α monoclonal antibodies (5, 30, and 50 ng per mL, R&D Systems), or the neutralizing bFGF monoclonal antibodies (10, 50, and 100 μg per mL, Upstate, Hamburg, Germany) for 48 h. Cells were then washed with PBS and medium was replaced by either serum-free RPMI or serum-free BLM cells conditioned medium in the presence of the antagonists and neutralizing antibodies. After 24 h, supernatants were collected and processed as described above.

For stimulation, human dermal fibroblasts (Fb; 5×10^4 cells per cm^2) were cultured in complete RPMI and in the absence or presence of recombinant TNF-α (20 ng per mL), EGF (30 ng per mL), bFGF (10 ng per mL), TGF-β1 (5 ng per mL), IL-6 (100 U per mL), IL-1α (10 pg per mL), activin (5 ng per mL), and PMA (0.4 nM) for 48 h (purchased from Tebu). Cells were then washed with PBS and incubated for 24 h in serum-free RPMI in the absence or presence of the cytokines and growth factors as described.

Immunoblot analysis For immunoblot analysis, serum-free conditioned media (corresponding to 20 μg protein) were separated on 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Hybond C, Amersham, Freiburg, Germany). After blockage of non-specific-binding sites with 5% non-fat milk in 0.5% Tween/PBS, blots were incubated with polyclonal anti-human MMP-1 antibody (diluted 1:2000) (Vallon *et al*, 1997), for 1 h at room temperature. Membranes were washed three times for 7 min in 0.5% Tween/PBS and incubated with porcine anti-rabbit antibody conjugated to horseradish peroxidase (1:2000 dilution, Dako Envision, Hamburg, Germany) for 1 h at room temperature. After three washes with 0.5% Tween/PBS solution, immunoreactive proteins were detected using the ECL western blotting detection reagents (Amersham) according to the manufacturer's instructions. All experiments were quantified by determining the signal intensities using ImageQuant software supplied to "Personal Densitometer" from Molecular Dynamics, Urbana, Illinois.

ELISA Fibroblasts and melanoma cell-conditioned media were assayed for protein levels of IL-1α and bFGF. ELISA was performed according to the manufacturer's instruction (human IL-1α and human bFGF, R&D Systems).

The relative amounts of cytokines and growth factors were calculated according to standard procedures.

Immunohistochemistry Cryosections (8 μm) of primary human melanoma were fixed in 4% paraformaldehyde in PBS for 15 min, washed twice with Tris-buffered saline (TBS), followed by blocking of non-specific-binding sites with 20% FCS/TBS. Primary antibodies were diluted in 10% FCS/TBS and, for IL-1α containing 0.5% saponin (Sigma, Taufkirchen, Germany). Sections were incubated with the primary antibody overnight at 4°C. After repetitive washing, bound antibodies were detected with an alkaline phosphatase-labelled anti-mouse/anti-rabbit polymer (Dako Envision) using neofuchsin as a substrate. Nuclei were counterstained with hematoxylin and mounted with Dako Faramount Aqueous Mounting Medium (Dako, Hamburg, Germany).

The following antibodies were used: anti-HMB-45 pre-diluted mouse monoclonal antibody IgG1 (Immunotech, Hamburg, Germany); mouse monoclonal antibody against LCA (clone 2B11; Dako Envision; final concentration 1:100); and monoclonal anti-human IL-1α antibody (Clone 4414.141, R&D Systems; final

concentration 10 μg per mL). Negative controls were performed by omission of primary antibodies.

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